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Incongruence between Morphological and Molecular Traits in Populations of *Viola violacea* (Violaceae) in Yamagata Prefecture, Northern Honshu, Japan

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Viola violacea is a morphologically variable and widely distributed species in Japan. *Viola violacea* var. *violacea*, distributed in western Japan, has narrowly ovate to widely lanceolate leaves, while *V. violacea* var. *makinoi* has lanceolate to narrowly lanceolate leaves and occurs in eastern Japan, but some populations of *V. violacea* var. *violacea* populations have been recorded in eastern Japan. Although the nature of these populations is obscure. We sought to uncover the origin of the disjunct *V. violacea* var. *violacea*-like populations in Yamagata Prefecture, Tohoku district by comparing leaf morphology, nuclear ribosomal DNA (nrDNA) ITS, and the *trnL–trnF* intergenic region of the chloroplast DNA (cpDNA). The leaf morphology of *V. violacea* var. *violacea*-like plants in Yamagata was identical to *V. violacea* var. *violacea* elsewhere, but the nrDNA ITS sequences of the Yamagata plants were more similar to *V. violacea* var. *makinoi*. This clear difference was not found in the cpDNA *trnL–trnF* region. The incongruity between leaf morphology and molecular traits may be due to *V. violacea* var. *violacea* populations that are more closely related to *V. violacea* var. *makinoi* or to intraspecific hybridization. To confirm these hypotheses, more extensive study is needed.

Key words: disjunct population, ITS, Japan, leaf morphology, PCA, phylogeny, *trnL–trnF*, variety, *Viola*

Viola violacea Makino (sect. *Adnatae* (W. Becker) V. V. Nikitin, Violaceae), is a small, acaulescent, perennial herb that grows on the margins and in the understory of forests in Japan, South Korea, and eastern China (Chen *et al.* 2007). In Japan, three varieties of *V. violacea* have been recognized, two of which are widely distributed across the Japanese archipelago. *Viola violacea* var. *violacea*, the nominal variety, with narrowly ovate to widely lanceolate leaf blades, is occurs in western Japan (Kinki and Chugoku districts of Honshu, Shikoku, and Kyushu), while variety *V. violacea* var. *makinoi* (H. Boissieu) Hiyama ex F. Maek., has lanceolate to narrowly lanceolate leaves and occurs in eastern Japan from the Tohoku district to the Kinki district (Hama 2002, Igari 2004; Fig. 1). The two varieties are distinguished primarily by leaf shape. Several additional morphological traits distinguish the two taxa. For example, the flowers are pale to deep reddish purple in var. *violacea* and deep purple in var. *makinoi* (Yamada 2010). The lower surface of the leaves is purple in var. *violacea* and slightly purple, becoming near green after flowering, in var. *makinoi* (Igari 2004). The expanding leaves of var. *violacea* are nearly horizontal while those of var. *makinoi* are nearly vertically oriented (Igari 2004). Variability in these traits, however, has been reported. For example, flower colors overlap according to the description of Ig-

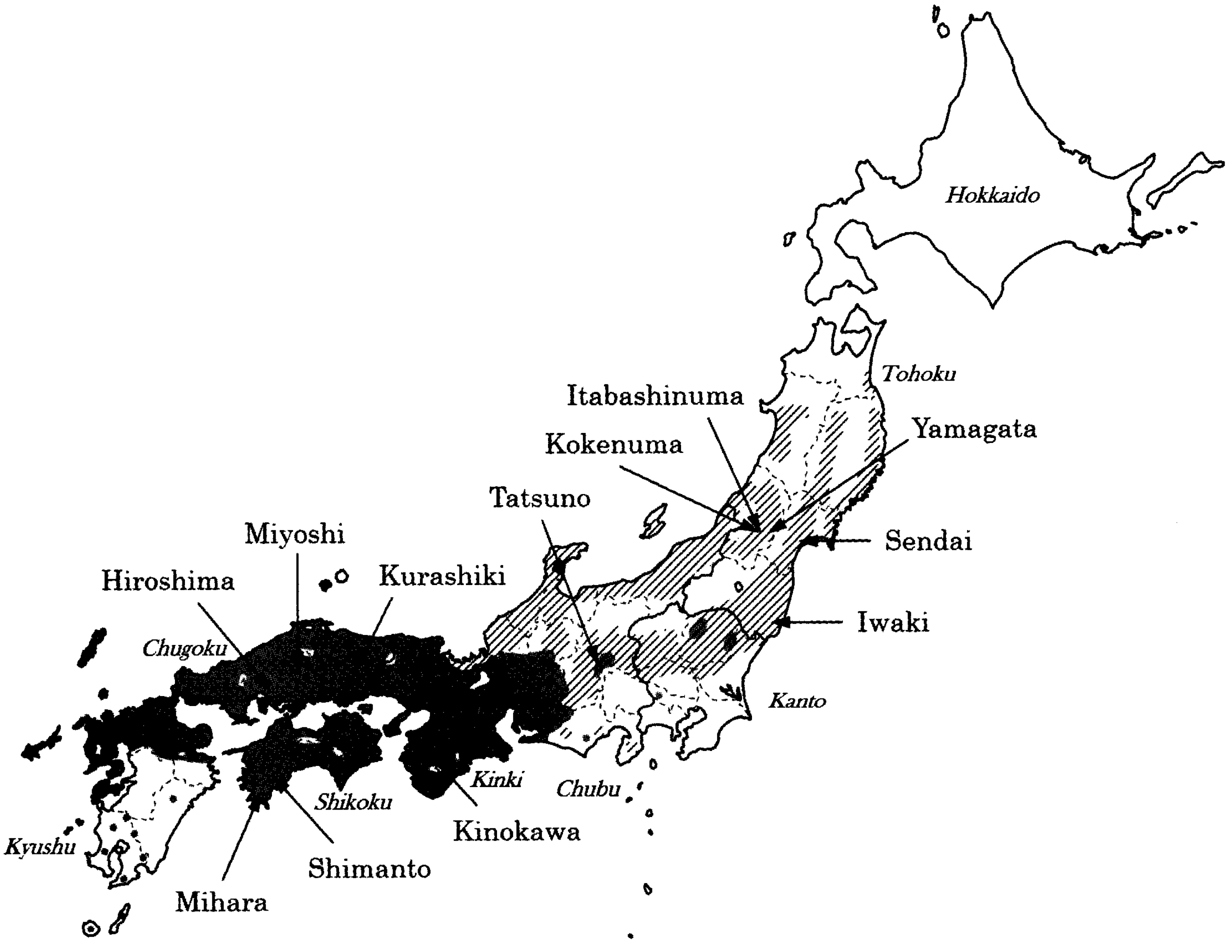

FIG. 1. Sampling locations with putative distribution ranges of two varieties of *Viola violacea* in Japan, based on Hama (2002). Shaded areas indicate distribution of *V. violacea* var. *violacea*; hatched area indicates distribution of var. *makinoi*.

ari (2004). Hama (2002) reported that both varieties usually have leaves with the lower surface purple. Expanded leaves are sometimes erect in var. *violacea* (M. Yoshida, personal observations). Leaf shape, therefore, is the most reliable characteristic for discriminating these two varieties.

Populations with intermediate morphological traits between the two varieties co-occur in parts of Kinki district and in some parts of eastern Japan (small areas in Tohoku, Kanto, and Hokuriku: Hama 2002, Igari 2004, Imai & Ito 2004, Yamada 2010). The status of these intermediate populations is unknown.

Takeda (2009) recognized several populations of var. *violacea* in the coastal region of southern Sanriku, Iwate Prefecture, Tohoku district, based on a morphological comparison of the leaves and flowers. His report is from an area far from the main distribution range of var. *violacea*. In Yamagata Prefecture, Tohoku district, populations that are indistinguishable from var. *violacea* have also been found (Hama 2002). The factors underlying the formation of the disjunct distribution of var. *violacea* remain obscure.

The process of current distribution pattern formation and cause of variation in morphological characteristics of *V. violacea* are poorly understood due to limited molecular phylogenetic study in Japan. Although molecular phylogenetic studies based on chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) sequences have been conducted on various species of *Viola* in eastern Asia (e.g., Yoo *et al.* 2005, Gong *et al.* 2010, Yoo & Jang 2010), few have focused on the phylogenetic relationships among the species endemic to Japan or on intraspecific taxa of *Viola.* The molecular basis of the intraspecific variation in *V. violacea* is unknown, and investigating the nature of the disjunct populations of var. *violacea* is important for uncovering the processes leading to the current distribution and morphological/genetic differentiation in the species.

To try to answer some of these questions, we focused on var. *violacea*-like populations in Yamagata Prefectural Forest, Yamagata Prefecture, northern Honshu, Japan. We compared the morphology and undertook molecular phylogenetic analyses to determine the status of the populations in relation to typical populations of the two varieties. Previous morphological comparisons of *V. violacea* (Takeda 2009), detected significant differences in leaf morphology, especially in blade width/length ratios, but not in flower traits. Our starting premise was that leaf shape is the best character for dividing the two varieties and would be appropriate for measuring morphological differentiation.

Materials and Methods

Plant materials were collected from July to September 2011. The sampling sites and numbers of individuals used for morphological measurements and molecular analysis are listed in Table 1. Sampling sites were selected where two varieties of *Viola violacea* with typical morphology were found. In this study, we included a variegated form of *V. violacea* var. *violacea* [f. *versicolor* in the sense of Hama (1975)] collected in Tatsuno, Nagano Prefecture. Nagano Prefecture is important because both varieties have been recorded from there (Hama 2002, Imai & Ito 2004; Fig. 1). The samples from Itabashinuma in the Yamagata Prefectural Forest, Yamagata, had variegated leaves and were identifiable as *V. violacea* f. *versicolor. Viola tokubuchiana* Makino var. *takedana* (Makino) F. Maek., which belongs to the same section as *V. violacea* (Hama 2002, Yoo & Jang 2010) collected in Shiroishi, Miyagi Prefecture, and Suwa, Nagano Prefecture, was used as the outgroup for phylogenetic analysis. Voucher specimens were deposited in the herbarium of Yamagata Prefectural Museum, Yamagata (YAMA).

Measurements of leaf morphology

One hundred individuals from nine populations were used for leaf shape measurements. For each individual, the largest leaf at collection time was selected for measurement. We measured blade length, blade width, length from the tip to the widest part of the blade, and length from the base to the widest part of the blade (Fig. 2). We also calculated the length-width ratio of the blade (= L1/L2) and the widest part ratio from the base of the blade (= L4/L1). Statistical analyses were conducted for the above measurements using Kruskal-Wallis nonparametric ANOVA tests followed by multiple comparisons with the nonparametric post hoc Steel-Dwass test. We also conducted principal components analysis (PCA) with an unbiased variance-covariance matrix to determine the overall tendency in the variation of leaf shape. Prior to analysis, the measurement data were transformed to z-scores. All analyses were conducted using R v.2.12.1 (R Development Core Team 2010).

DNA extraction, sequencing, and phylogenetic analysis

Total DNA was extracted from dried leaves held in silica gel using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The PCR reaction solution was prepared with a total volume of 50 μL containing 1.25 units of *TaKaRa Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan), 5 μL 10× *Ex Taq* Buffer (2 mM final Mg^{2+} concentration; Takara Bio Inc.), 0.2 mM of each dNTP Mixture (Takara Bio Inc.), 0.5 μM of each primer, and ca. 100 ng of total DNA. We used the universal primers "ITS5" and "ITS4" (White *et al.* 1990) to amplify the internal transcribed spacer (ITS) region of nrDNA and "e" and "f" (Taberlet *et al.* 1991) to amplify the *trnL–trnF* intergenic region of cpDNA. The following program was used for amplification: one cycle at 95°C (3 min), followed by 35 cycles at 95°C (30 s), 51°C (30 s), and 72°C (90 s), with a final 5 min extension at

TABLE 1. Localities and number of individuals collected as samples in this study.

Species	Locality	Number of individuals		Accession number	
		Leaf morphology	Molecular analysis	ITS	*trnL–trnF*
Viola violacea var. *violacea*	Hiroshima city, Hiroshima Pref., Japan	12	2	AB759231, AB759232, AB759233, AB759234	AB759266, AB759267
	Miyoshi city, Hiroshima Pref., Japan	17	2	AB759235, AB759236, AB759237, AB759238	AB759268, AB759269
	Kurashiki city, Okayama Pref., Japan	12	2	AB759239, AB759240, AB759241, AB759242	AB759270, AB759271
	Shimanto city, Kochi Pref., Japan	–	1	AB759243, AB759244, AB759245	AB759272
	Mihara vil., Kochi Pref., Japan	–	1	AB759246	AB759273
	Kinokawa city, Wakayama Pref., Japan	–	1	AB759247	AB759274
	Cheju Island, South Korea	–	1	AY928308	DQ085921
	Tatsuno town*, Nagano Pref., Japan	18	2	AB759248, AB759249, AB759250, AB759251	AB759275, AB759276
V. violacea var. *makinoi*	Sendai city, Miyagi Pref., Japan	6	2	AB759252, AB759253	AB759277, AB759278
	Yamagata city, Yamagata Pref., Japan	9	2	AB759254, AB759255	AB759279, AB759280
	Iwaki city, Fukushima Pref., Japan	9	2	AB759256, AB759257	AB759281, AB759282
V. violacea var. *violacea*-like in Yamagata	Itabashinuma*, Yamagata Prefectural Forest, Yamanobe town, Yamagata Pref., Japan	8	2	AB759258, AB759259	AB759283, AB759284
	Kokenuma, Yamagata Prefectural Forest, Yamagata city, Yamagata Pref., Japan	9	2	AB759260, AB759261, AB759262, AB759263	AB759285, AB759286
Outgroup					
Viola tokubuchiana var. *takedana*	Shiroishi city, Miyagi Pref., Japan	–	1	AB759264	AB759287
	Suwa city, Nagano Pref., Japan	–	1	AB759265	AB759288

*: *Viola violacea f. versicolor*

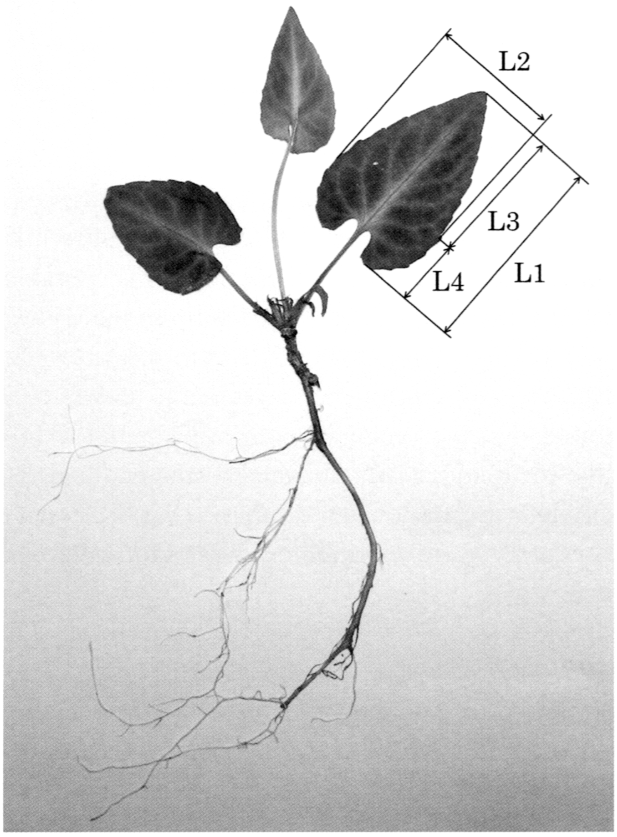

FIG. 2. Measurements of leaf morphology. Specimen is a *Viola violacea* var. *violacea*-like plant from Yamagata (Kokenuma). L1: blade length. L2: blade width. L3: length from tip to widest part of blade. L4: length from base to widest part of blade.

72°C. The products were purified using a QIAEXII Gel Extraction Kit (Qiagen). Cycle sequencing was performed using a BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with primers used for PCR amplification. The product was purified using an illustra Autoseq G-50 Dye Terminator Remover Kit (GE Healthcare, Milwaukee, WI, USA). The sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For samples with overlapping wave signals in the electrophoretic pattern in the ITS region, we determined the nucleotide sequences a second time, but reduced the PCR thermal cycles to 30. With these data, we confirmed whether the overlapping signals were attributable to artificial PCR errors. The PCR samples with several polymorphic nucleotides were purified as described above, and cloned with the TA Cloning Kit using One Shot TOP10 *E. coli* (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

The obtained sequences were aligned using ClustalW (Thompson *et al.* 1994) for the ITS and using MUSCLE (Edgar 2004) for the *trnL–trnF* region sequences with *V. violacea* from Cheju Island (accession number AY928308 and DQ085921) in MEGA v.5.05 (Tamura *et al.* 2011). Each sample with a polymorphic nucleotide position was divided into two separate sequences and both were included in the following analyses.

To visualize the haplotype relationship of the ITS and *trnL–trnF* regions, we constructed a statistical parsimony network using TCS v.1.21 (Clement *et al.* 2000). We postulated that each indel occurred only once, and treated it as a fifth state. Phylogenetic trees were constructed by the maximum parsimony (MP) and maximum likelihood (ML) method. The indels were treated in same way as the parsimony network in MP analysis and designated as missing data in ML analysis. The parsimony analyses were conducted with 100 rounds of random sequence additions and the tree-bisection-reconnection (TBR) branch swapping algorithm using PAUP* v.4.0b10 (Swofford 2002). The ML analysis was conducted using Treefinder, version of March 2011 (Jobb *et al.* 2004, Jobb 2011). The best-fit nucleotide substitution models for ITS and *trnL–trnF* regions were selected based on the Akaike information criterion (Akaike 1974). The J2+G model (estimated nucleotide frequency: T 0.185, C 0.309, A 0.202, G 0.305; estimated substitution rates: TC 0.459, TA = CA 0.100, TG = CG 0.037, AG 0.267; α = 0.176) was selected for the ITS region sequence; the HKY model (estimated nucleotide frequency: T 0.349, C 0.173, A 0.309, G 0.169; estimated substitution rates: TC = AG 0.300, TA = TG = CA = CG 0.100) was selected for the *trnL–trnF* region. To test the reliability of the MP and ML trees, 1000 bootstrap replications (Felsenstein 1985) were performed.

Results

Leaf morphology

The measurements and ratios calculated from them were compared among three groups: *Viola violacea* var. *violacea*, *V. violacea* var. *makinoi*, and *V. violacea* var. *violacea*-like populations in Yamagata. Significant differences were noted between var. *makinoi* and the other two groups in blade width, length from the base to the widest part of the blade, length-width ratio and the widest part ratio from the base of the blade (Fig. 3). No significant differences were found in any measurements or ratios between var. *violacea* and the var. *violacea*-like populations in Yamagata. *Viola violacea* var. *makinoi* has narrower leaves than the other two groups (median length-width ratio of the leaf blade of var. *violacea* = 1.57; var. *violacea*-like populations in Yamagata = 1.62; and var. *makinoi* = 2.25). The widest part of the blade in var. *makinoi* was closer to the base than in the other two groups (median value for var. *violacea* = 0.23; var. *violacea*-like populations in Yamagata = 0.23; and var. *makinoi* = 0.15).

The first principal component of the PCA explained the size of the leaf blade as a whole, while the second principal component incorporated blade width and length from the tip to the widest part of the blade (Table 2). From a scatterplot based on the principal component scores, we found that the range variation of var. *violacea*-like populations in Yamagata overlapped considerably with that of var. *violacea* (Fig. 4). Variation in leaf size was greater in var. *violacea* than in any other group.

Molecular analysis

We examined 654 bp from the ITS region and 402 bp from the *trnL–trnF* region for each sample. In the ITS region, heterozygous sites were

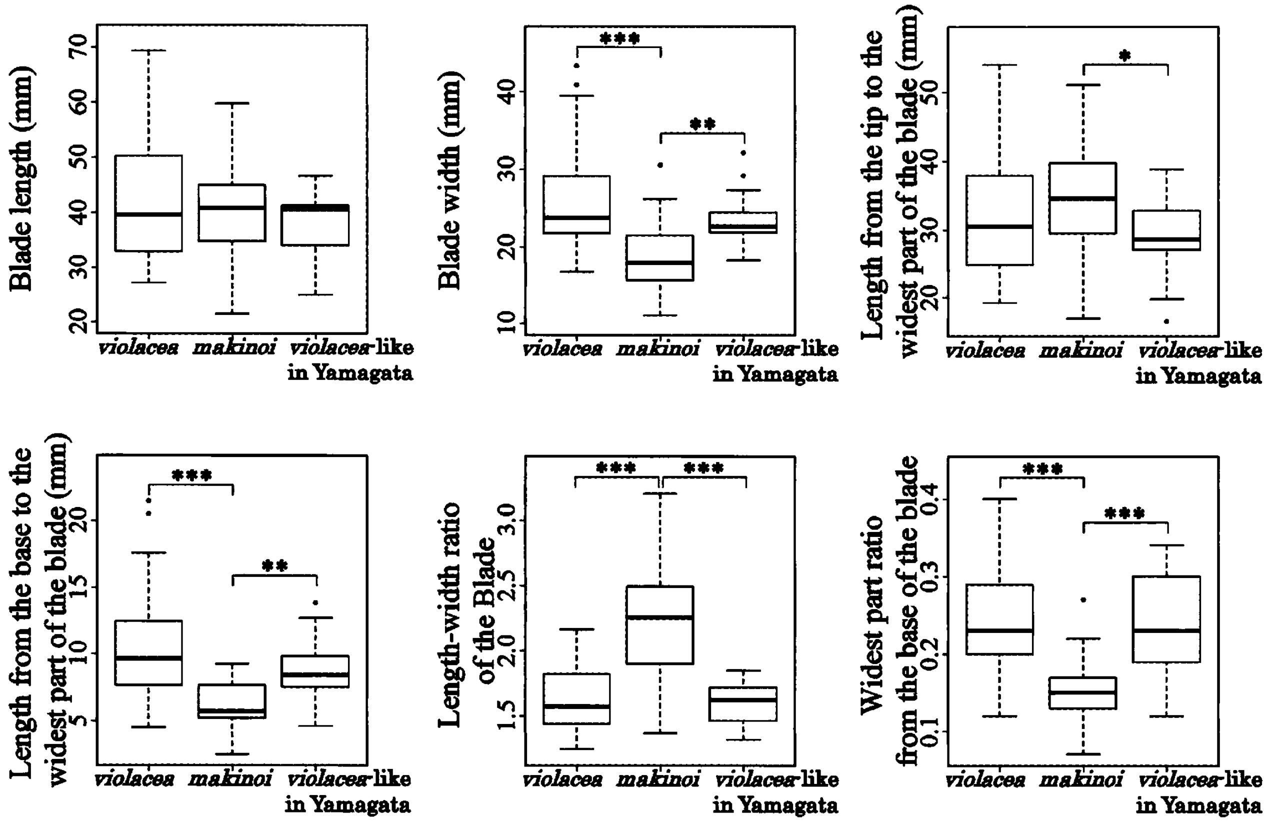

FIG. 3. Box plots for each measurement and calculated ratios for leaf morphology of *Viola violacea*. Significant differences detected between groups by post hoc Steel-Dwass tests are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

TABLE 2. Loading of principal components.

	PC1	PC2
Blade length	-0.31	0.06
Blade width	-0.26	-0.24
Length from the tip to the widest part of the blade	-0.29	0.15

detected in six populations (Hiroshima, Miyoshi, Kurashiki, Shimanto, Tatsuno, and Kokenuma) and cloned PCR products were obtained from two of them (Kurashiki and Shimanto). All heterozygous sites were also detected when we reduced the PCR thermal cycles to 30, confirming that the overlapping signals were not due to artificial PCR errors. We observed no substitutions or heterozygous sites among the samples of var. *makinoi*. Synapomorphic substitutions in var. *violacea*-like populations in Yamagata were found at two sites (618 and 626 bp alignment positions; Table 3). The samples from Shimanto and Cheju Island had large deletions (227 bp: 118–344 bp) in the *trnL–trnF* intergenic region. *Viola violacea* var. *violacea*-like populations in Yamagata had a four-base deletion (330–333 bp) as a synapomorphic character.

An analysis of a statistical parsimony network based on the ITS sequences showed that haplotypes of var. *violacea*-like populations in Yamagata were close to those of var. *makinoi* and not directly connected to any haplotypes of var. *violacea* (Fig. 5a). Various haplotypes were found in var. *violacea*, but only a single haplotype was found in var. *makinoi* (Table 3). The haplotypes of the Hiroshima, Miyoshi and Cheju Island populations were far apart from most of other ITS haplotypes. Haplotype Q–T were cloned sequences obtained from Kurashiki (Q–S) and Shimanto (T), and are placed in positions distant from other haplotypes. The network of the *trnL–trnF* intergenic region showed that the two varieties were indistinguishable by haplotype distribu-

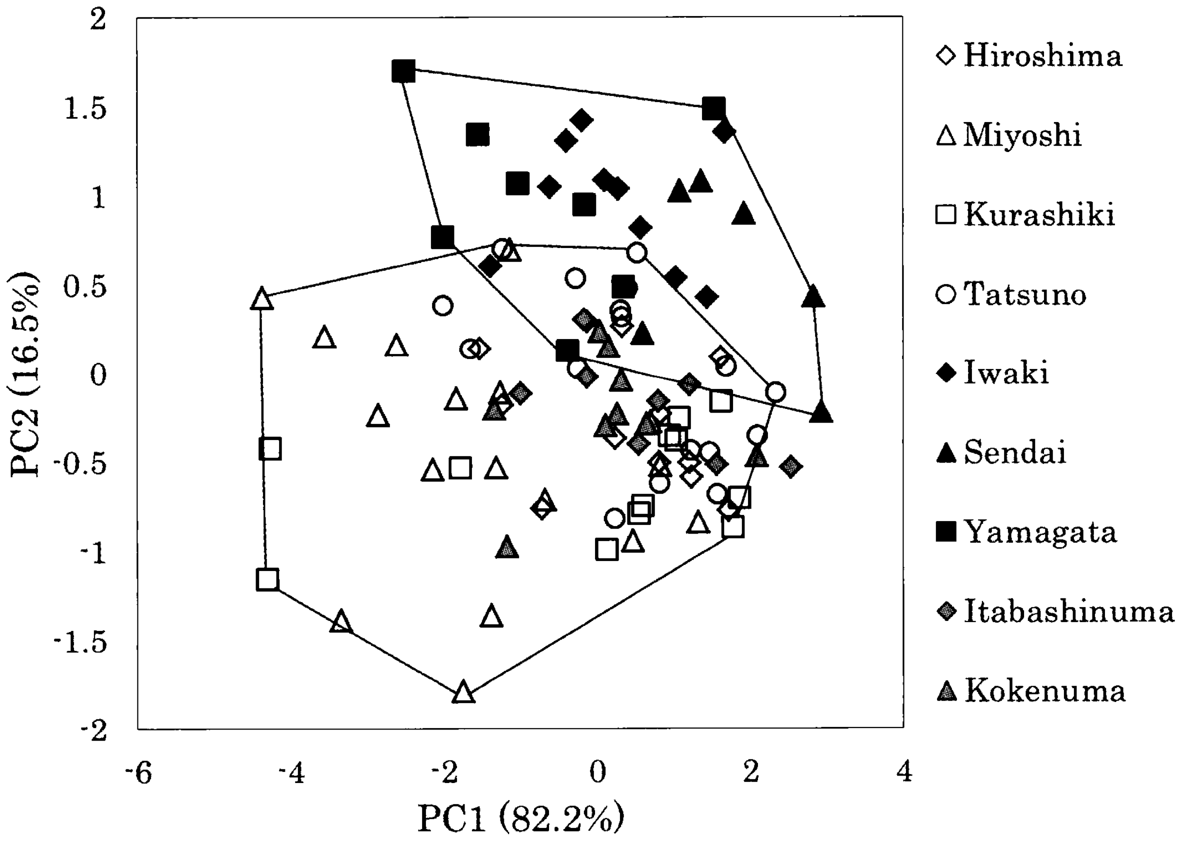

FIG. 4. Scatterplot of PCA scores for three measurements of leaf morphology. The contribution of each axis is indicated beside axis name. Color symbols corresponds to group: *Viola violacea* var. *violacea*, white; *V. violacea* var. *makinoi*, black; *V. violacea* var. *violacea*-like population in Yamagata, gray.

TABLE 3. Variable sites in the 654 bp of the ITS region of *Viola violacea*.

Haplotype	Variable site																																									Localities (variety)
	28	47	59	62	66	75	83	85	90	95	97	104	105	110	131	133	140	142	166	167	168	180	184	199	218	231	234	241	392	447	453	459	464	465	473	501	580	609	611	618	626	636	
A	C	C	C	G	C	C	C	T	C	T	G	A	A	T	T	T	C	G	A	C	T	C	G	G	G	T	T	C	T	A	A	T	A	T	G	C	C	A	G	C	A	G	Shimanto (v)
B	.	.	.	.	.	.	.	C	.	C	.	T	G	.	C	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	**Kurashiki (v)**
C	.	.	T	.	.	.	.	C	.	C	.	T	G	C	C	C	.	.	.	.	.	.	.	.	.	C	C	.	.	T	G	.	.	.	.	.	.	.	.	.	.	C	Mihara (v)
D	.	.	T	.	.	.	.	C	.	C	.	T	G	C	C	C	.	.	.	.	.	.	.	A	A	C	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	C	**Kinokawa (v)**
E	.	.	T	.	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	A	.	.	.	.	.	C	T	.	.	T	G	.	.	.	.	.	.	.	C	Tatsuno (v)
F	.	.	T	.	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	.	.	.	.	.	.	C	T	.	.	T	G	.	.	.	G	A	.	.	C	**Tatsuno (v)**
G	.	.	T	A	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	C	.	.	.	.	.	.	.	C	T	.	.	T	G	.	.	.	G	A	.	.	C	Tatsuno (v)
H	.	.	T	A	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	C	.	A	.	.	.	.	.	C	T	.	.	T	G	.	.	.	.	.	.	.	C	**Tatsuno (v)**
I	.	.	T	A	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	A	.	.	.	.	.	C	T	.	.	T	G	.	.	.	.	.	.	.	C	Sendai (m), Yamagata (m), Iwaki (m)
J	.	.	T	A	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	A	.	.	.	.	.	C	T	.	.	T	G	.	.	.	.	.	A	C	C	**Kokenuma (vl)**
K	.	.	T	A	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	A	.	.	.	.	.	C	T	.	.	T	G	A	.	.	.	.	A	C	C	Kokenuma (vl)
L	.	.	T	.	.	.	.	C	.	.	.	T	G	C	C	C	.	.	C	A	.	.	.	.	.	.	.	.	C	T	.	.	T	G	.	.	.	.	.	A	C	C	**Itabashinuma (vl)**
M	T	T	T	.	T	A	A	C	T	.	T	T	.	C	.	.	T	A	C	A	.	A	A	.	.	.	.	.	.	T	.	.	T	G	.	T	.	.	.	.	.	C	Miyoshi (v), Cheju Island (v)
N	T	T	T	.	T	A	A	C	T	.	T	T	.	C	.	.	T	A	C	A	.	A	A	.	.	C	.	.	.	T	.	C	T	G	.	T	.	.	.	.	.	C	**Miyoshi (v)**
O	T	T	T	.	T	A	A	C	T	.	T	T	.	C	.	.	T	A	C	A	.	A	A	.	.	.	.	.	.	T	.	.	T	G	.	T	A	.	.	.	.	C	Hiroshima (v)
P	T	T	T	.	T	A	A	C	T	.	T	T	.	C	.	.	T	A	C	A	.	A	A	.	.	.	.	.	.	T	.	.	T	G	.	.	.	.	.	.	.	C	**Hiroshima (v)**

The plural haplotypes in same locality could explain presence of heterozygous site. The dots in list represent same base to the first row. A character in parenthesis beside locality name stand for each varieties (v: var. *violacea*, m: var. *makinoi*, vl: var. *violacea*-like in Yamagata).

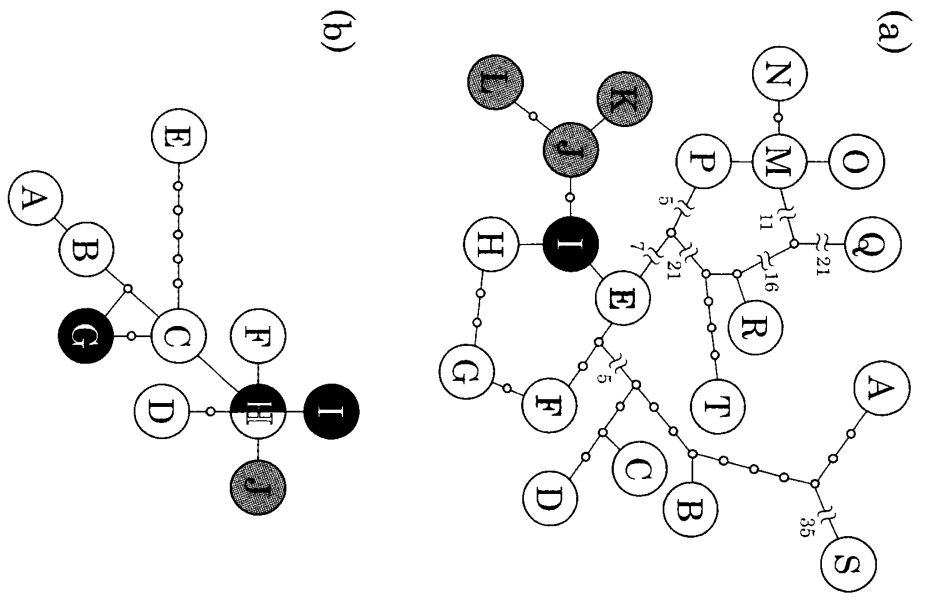

FIG. 5. Statistical parsimony network based on ITS region sequences with fixed connection limit at 39 step (a) and *trnL–trnF* region sequence with a 95% connection limit setting (b). Color of each symbol corresponds to group: *Viola violacea* var. *violacea*, white; *V. violacea* var. *makinoi*, black; *V. violacea* var. *violacea*-like population in Yamagata, gray. Small open circle represents haplotypes not appearing in analysis and connections between haplotypes with five or more steps represent "≈" with number of steps.

tion (Fig. 5b). All samples from var. *violacea*-like populations in Yamagata had the same haplotype, which was connected to haplotype H. Haplotype H was shared by var. *violacea* in Tatsuno and var. *makinoi* in Sendai. Haplotype E of var. *violacea* in Hiroshima was very different from the other haplotypes of *V. violacea*, and a BLAST search (Altschul *et al.* 1997) indicated this haplotype is from another species in sect. *Adnatae*. For this reason, we did not include haplotype E in Table 4.

The phylogenetic trees of ITS sequences (Fig. 6; MP: 78 trees; tree length = 144; CI = 0.79; RI =

0.91; ML: ln likelihood = –1732.026) indicated the paraphyletic arrangement of var. *violacea* and the derivation of var. *makinoi* from var. *violacea*. The populations of var. *violacea*-like plants in Yamagata were more closely related to var. *makinoi* and to var. *violacea* in Tatsuno, Nagano Prefecture. In the MP tree, var. *violacea* samples from Hiroshima, Miyoshi and Cheju Island formed a monophyletic group (76% bootstrap value), but this group was not supported by the ML analysis. The trees based on the *trnL–trnF* sequence (Fig. 7; MP: 12 trees; tree length = 20; CI = 0.95; RI = 0.98; ML: ln likelihood = –608.0161) had less resolution than those based on ITS data. The OTU arrangement of the *trnL–trnF* sequence trees was different from that of ITS trees, and no apparent phylogenetic groups corresponded to varieties of *V. violacea*. Populations of var. *makinoi* were split; the Iwaki population appeared at a position that was relatively basal in the trees. Although the var. *violacea*-like plants in Yamagata and var. *makinoi* in Yamagata formed a weakly supported monophyletic group in the MP tree (62%, 63% bootstrap values respectively), the group was not supported in the ML tree (<50%).

Discussion

Origin of Viola violacea *var.* violacea*-like plants in Yamagata*

As indicated previously (e.g., Hama 2002, Igari 2004), typical *Viola violacea* var. *makinoi* has narrower leaves and can be distinguished from *V. violacea* var. *violacea* by leaf morphology. Based on an analysis of leaf morphology, the *V. violacea* var. *violacea*-like plants in Yamagata were identified as var. *violacea*. From our phylogenetic analysis of ITS sequences, however, the var. *violacea*-like plants in Yamagata are included in the lineage mostly composed of var. *makinoi*. The network analysis of ITS sequences also indicated that var. *violacea*-like plants in Yamagata are closely related to var. *makinoi*. The *trnL–trnF* sequences did not clearly differentiate between varieties; var. *violacea*-like plants in Yamagata were derived from the haplotype possessed by

TABLE 4. Variable sites in the 402 bp of the *trnL–trnF* intergenic region of *Viola violacea*.

Haplotype	Variable site											Localities (variety)
	61	104	162	175	244–248	259	274	275–282	326–327	330–333	355	
A	C	C	—	G	—	—	A	GATAAGAC	—	AGCC	A	Miyoshi (v), Kurashiki (v)
B	.	.	.	.	.	.	.	.	.	.	G	**Mihara (v)**
C	.	A	—	—	—	—	—	—	—	—	G	Shimanto (v), Cheju Island (v)
D	.	.	T	A	.	.	.	—	CA	.	G	**Hiroshima (v)**
F	T	A	T	A	.	.	.	—	.	.	G	Kinokawa (v)
G	.	.	.	.	.	C	G	.	.	.	G	**Iwaki (m)**
H	.	A	T	A	.	.	.	—	.	.	G	Tatsuno (v), Sendai (m)
I	.	A	T	A	TTTTA	.	.	—	.	.	G	**Yamagata (m)**
J	.	A	T	A	.	.	.	—	.	—	G	Itabashinuma (vl), Kokenuma (vl)

The haplotype of Shimanto and Cheju Island have deletion from 118 to 344 bp. The dots in list represent same base to the first row and gaps are "—". A character in parenthesis beside locality name stand for each varieties (v: var. *violacea*, m: var. *makinoi*, vl: var. *violacea*-like in Yamagata).

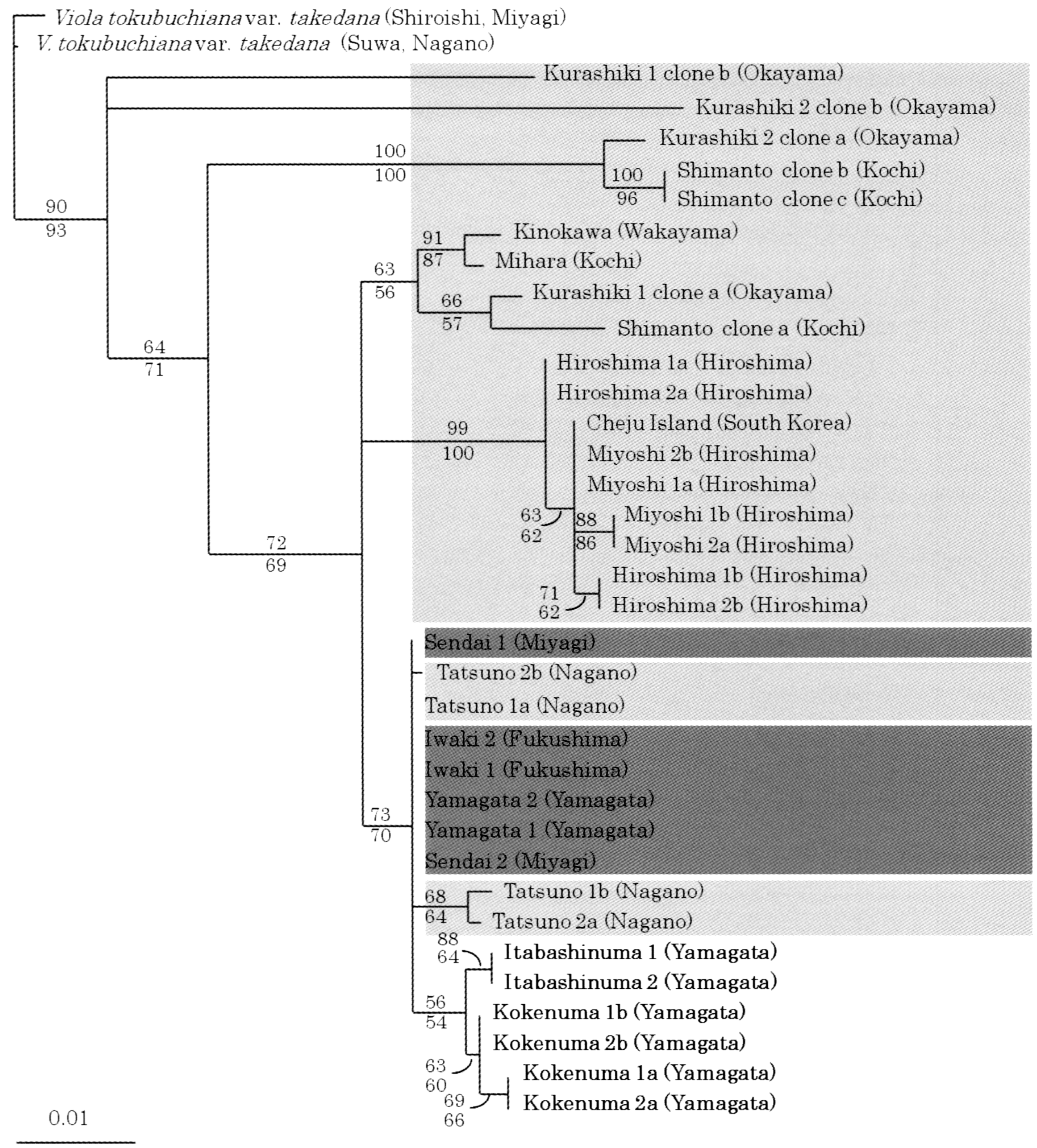

FIG. 6. Maximum likelihood (ML) phylogenetic tree based on ITS region sequences of *Viola violacea*. Sequences obtained from *V. violacea* var. *violacea* (light gray) and *V. violacea* var. *makinoi* (dark gray) are highlighted. Samples of *V. violacea* var. *violacea*-like population in Yamagata are shown in boldface. Numbers following locality names discriminate different individuals; some are followed by letters (a, b, or c) indicating discrimination of sequences obtained from same sample by manual separation or by cloning. Prefecture names of localities are shown in parentheses. Bootstrap values (>50%) of ML (above branches) and maximum parsimony (below branches) are shown (each with 1000 replicates).

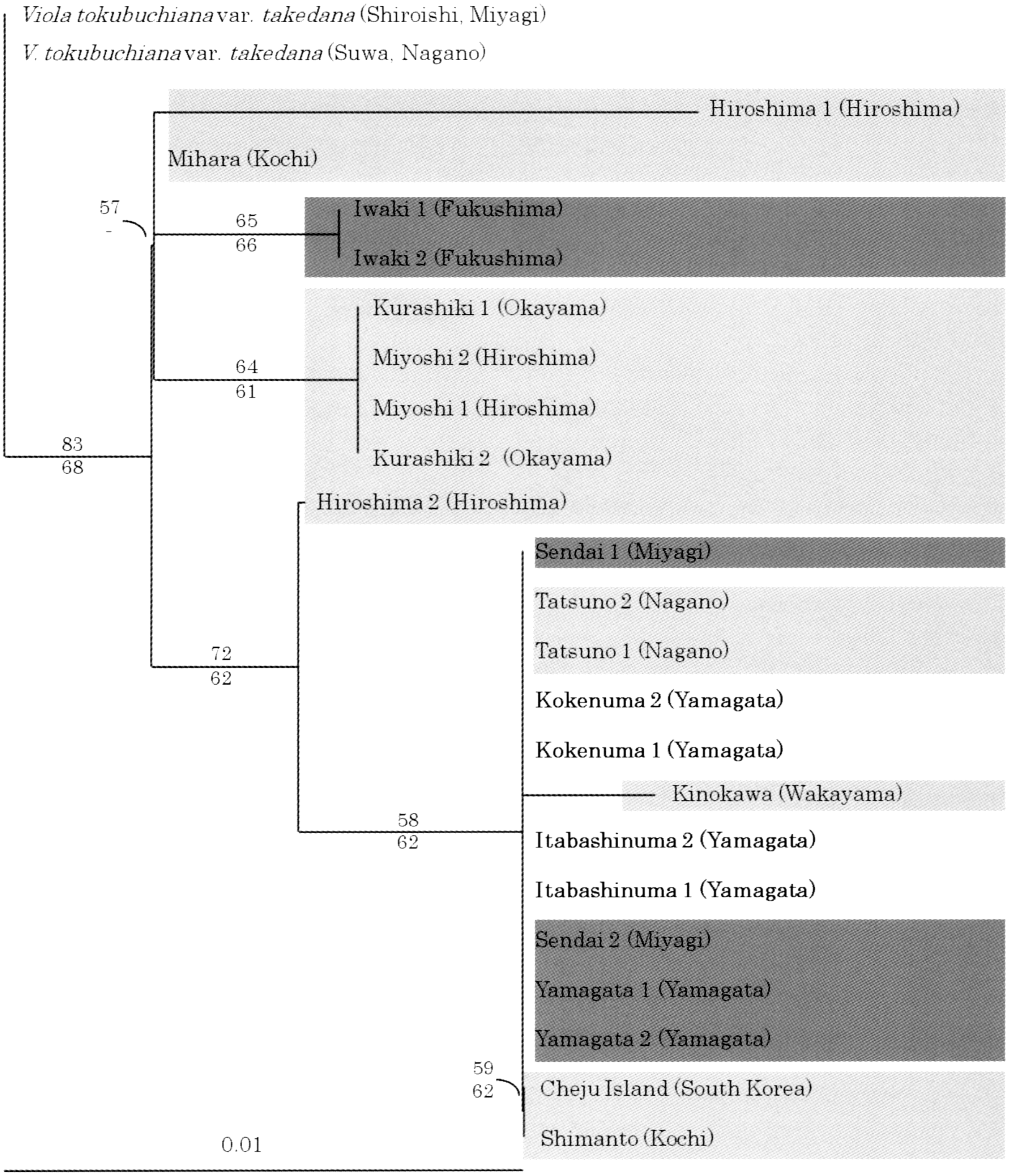

FIG. 7. Maximum likelihood (ML) phylogenetic tree based on *trnL–trnF* region sequences of *Viola violacea*. Sequences obtained from *V. violacea* var. *violacea* (light gray) and *V. violacea* var. *makinoi* (dark gray) are highlighted. Samples of *V. violacea* var. *violacea*-like population in Yamagata are shown in boldface. Numbers following locality name discriminate different individuals. Prefecture names of localities are shown in parentheses. Bootstrap values (>50%) of ML (above branches) and maximum parsimony (below branches) are shown (each with 1000 replicates).

both varieties. Thus, var. *violacea*-like plants in Yamagata were genetically different from typical var. *violacea* in western Japan and more closely related to var. *makinoi*.

Two explanations for the origin of var. *violacea*-like plants in Yamagata can be considered in the light of results from this study. First, *V. violacea* is genetically differentiated between western and eastern Japan. Our phylogenetic and network analyses of both ITS and *trnL–trnF* sequences indicated that the eastern var. *violacea* identified from leaf morphology in the Yamagata Prefectural Forest and Tatsuno was genetically differentiated from typical var. *violacea* in western Japan and more closely related to var. *makinoi*. In this scenario, populations of var. *violacea* might have expanded their distribution range to the east and become scattered over several parts of eastern Japan, where they were isolated from the western populations. One of the eastern var. *violacea* phylogenies differentiated into var. *makinoi* while the others remained morphologically identifiable as var. *violacea*. The second possible explanation is a hybrid origin of the Yamagata plants. Based on our measurements, the leaf morphology of the plants from Yamagata were within the range of variation of var. *violacea*, but their ITS sequences were closer to var. *makinoi*. This disparity may have resulted from past hybridization between the two varieties of *V. violacea*. Because of their close phylogenetic positions to var. *makinoi*, both the Yamagata plants and var. *violacea* in Tatsuno could have originated through hybridization.

Due to the concerted evolution of ribosomal DNA, support for hybridization and introgression may be lacking, given a phylogenetic relationship based on ITS sequences (Álvarez & Wendel 2003). In such cases, applying single- or low-copy nuclear genes is recommended, and those genes can be used as phylogenetic markers to identify evolutionary relationships between species that have experienced interspecific hybridization (van den Hof *et al.* 2008, Marcussen *et al.* 2011, 2012). To confirm these scenarios, more extensive surveys of var. *violacea* (including morphologically intermediate populations) in eastern Japan and the application of various nuclear markers should be conducted.

Phylogenetic relationship between two varieties of Viola violacea

The phylogenetic relationship between the two varieties of *Viola violacea* indicated that var. *violacea* is genetically differentiated into several lineages, including var. *makinoi*. No substitutions or polymorphic sites among the ITS sequences in some populations of var. *makinoi* may indicate a recent origin of the varieties. These phylogenetic interpretations are congruent with previous morphological insights that regard var. *makinoi* as a narrower leaf variation of var. *violacea* (e.g., Momiyama 1982, Hama 2002, Igari 2004). Haplotype differentiation between the two varieties of *V. violacea*, however, was not obvious in the cpDNA *trnL–trnF* intergenic region, and three haplotypes were found in var. *makinoi*. Higher haplotype diversity in cpDNA than in the ITS of var. *makinoi* may be explained by lineage sorting with ancestral polymorphism or past hybridization between two varieties, as discussed above. At the moment, we do not have sufficient information to determine which of the possible scenarios provides a better explanation of the facts. The finding of an individual with cpDNA from different *Viola* species in the Hiroshima population suggests the possibility of a past hybridization event; at least the Iwaki population is distantly related to other populations of var. *makinoi* in cpDNA.

Viola has been reported to produce interspecific hybrids easily (e.g., Conesa *et al.* 2008, van den Hof *et al.* 2008, Marcussen *et al.* 2011). Hybridization may be an important factor to explain the various levels of diversity among the species of *Viola*. If the cpDNA diversity in var. *makinoi* is explainable by chloroplast capture as results of past hybridizations, the simple genetic composition seen in the ITS region may indicate rapid range expansion of var. *makinoi* into the northeastern part of Honshu. Current information, however, is inadequate for tracing the processes of differentiation and distribution in *V. violacea*. Additional populations should be surveyed to explore these processes in detail.

Many ecological or physiological adaptations to the environment in eastern Japan (e.g., cool-temperate climate) may relate to the rapid expansion of var. *makinoi*. *Viola violacea* var. *makinoi* is mentioned by some authors to tolerate more severe drought conditions than var. *violacea* (Hama 2002, Igari 2004, Takeda 2009). If so, it suggests an influence of climatic factors in shaping the present distribution pattern of varieties of *V. violacea*; the habitats of the two varieties (var. *makinoi* and var. *violacea*) might be determined by subtle conditions (e.g., air humidity or water retention of soil). The morphological differentiation and growth habit of the leaves might also reflect an ecological adaptation. Including the Yamagata populations described above, two varieties of *V. violacea* are undergoing a complicated differentiation process (Igari 2004). Thus, extensive studies, including ecological, physiological, and genetic, should be conducted to examine the overall differentiation process among widespread species of violets in the Japanese Archipelago.

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